

METHOD FOR PERFORMING MULTIPLEX PCR WITH CYCLIC CHANGES OF PCR PARAMETERS

BACKGROUND OF THE INVENTION

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FIELD OF THE INVENTION

The present invention relates to an improved method for multiplex PCR for resulting in at least two amplified DNA products from one PCR solution. In particular, the present invention relates to the novel method for performing multiplex PCR to solve the problems that size differences among many types of required amplified products can not be greatly increased, non-specific DNA amplified products are produced, and dimers caused by using at least two pairs of primers in the conventional multiplex PCR.

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DESCRIPTION OF THE PRIOR ART

To perform disease diagnosis, detection and identification of pathogenic bacteria, and research on variation or abnormality of organism gene, such as SNP(single nucleotide polymorphism), STR(short tandem repeat), insertion, deletion and mutation etc, the sequence of amplified target DNA must be secured in advance. As a method for amplifying target DNA sequence, there exists PCR (polymerase chain reaction) method, SDA(strand displacement amplification) method, and ones for TMA(transcription mediated amplification), by which the target DNA is synthesized from mRNA, NASBA(nucleic acid sequence based amplification) and so on. In general

applications, the PCR method is widely used for its amplification efficiency superior to other methods above.

In recent years, researches have been conducted to perform DNA amplification in a small equipment or a small chip like a lab-on-a-chip and to
5 analyze DNAs in a short time

However, common problems of the above methods are have common problems as follows. It is complicated to purely refine genomes from blood samples or tissue samples, and various target genes must be amplified safely in a small space like chips in order to obtain information as many as possible
10 with small costs. Multiplex PCR method have been used to obtain various DNA amplified products in a limited space.

The multiplex PCR method produces at least two amplified DNA products from one reaction solution, and at least two pairs of primers are used. Therefore, if the reaction condition and primer sequence are not appropriate,
15 primer dimers may be produced or non-specific DNA products may be produced, or required-amplified DNA products can not be obtained. Conventional methods require a lot of times and efforts in order to find the optimal condition for amplification of different size of DNA because temperature and time conditions are fixed. For example, in the documents by
20 Hinegariu O et al, "Multiplex PCR: critical parameters and step-by-step protocol"(Biotechniques, 1997, pp. 504-511) and by P. markoulatos et al, "Multiplex Polymerase Chain Reaction: a practical approach" (J. Clin. Lab. Anal. 2002. pp. 47-51), researches have been conducted to find the optimal multiplex PCR conditions for various combination of PCR parameters like

concentration of primers, Mg⁺⁺, taq DNA polymerase buffer, temperature of annealing and extension time to a specific sample. However, these conventional methods have some problems, such as, the methods require long time, much efforts and large amount of samples to optimize the PCR condition
5 because of various combinations of PCR parameter, and the procedures should be repeated for a new samples.

SUMMARY OF THE INVENTION

Therefore, the object of the present invention is to provide a method
10 for finding optimal multiplex PCR condition for performing simple and stable PCR.

Meanwhile, another object of the present invention is to provide the method for multiplex PCR using a sample which has been required to perform the conventional multiplex PCR, especially a diluted blood sample which has
15 not been refined in the case of a blood sample.

To achieve the above objects, inventors of the present invention have conducted researches and have found that target DNA sequences can be amplified in simple and safe way by the multiplex PCR method of the present invention by changing annealing temperature and extension time among
20 several parameters of the multiplex PCR per cycle having constant period.

Further, a pretreatment step of a raw sample like blood, which has been diluted by buffers and not been refined, can be directly used in a multiplex PCR, thereby a preconditioning step for obtaining refined DNAs can be eliminated.

Also, DNA can be amplified in multiplex PCR with a small amount of about 1 μl of reaction volume.

As a result of recent development in molecular biology, not only human genome sequences are completely analyzed but genome sequences of
5 organism useful to a human being or valuable for research are subsequently analyzed and disclosed. To be meaningful and to be used by a human-being, some genes of the disclosed sequences expressing protein and function of the genes should be revealed. In recent years DNA chip related fields are developing fast to solve the above tasks. Researches are being conducted to
10 find functions of unknown genes from gene expression patterns of patients having specific disease, or from gene expression patterns responding to stimulus from outside of a living body. Meanwhile, as the genome researches proceed, it has been found that sequences between individuals are slightly different even in the gene of which functions and sequences are
15 disclosed. Such differences of base sequences present between individuals or species constituents are called SNP. The SNP not only causes differences of character between individuals but becomes one reason for differences of resistance and sensitiveness to drugs, and in the worst case cause of genetic diseases. Many laboratories have built SNP database from known human
20 genomes and been making an effort to find out relationship between drug sensitiveness and disease to the SNP.

In the future, accumulated gene related information will be used in a development of DNA diagnosis equipment. For the purpose of gene diagnosis, refined genomes should be prepared from samples and DNA of

interest should be amplified. These tasks become obstacles to miniaturization and speed-up of diagnosis equipment and fast speed. According to the present invention, to obtain various kinds of amplified DNA products by the multiplex PCR, blood sample is directly used for DNA
5 amplification, and the above problems can be overcome.

The present invention performs multiplex PCR by using either of raw blood sample or refined DNA.

There exists a need for PCR equipment capable of programming adjustment of temperature and other reaction conditions for multiplex PCR.
10 The PCR comprises steps of preliminary denaturing, denaturing, cycling (annealing and extension), and additional extension and the PCR program consists of set values of temperature and time of each step and number of cycle of the cycling step. The method for performing PCR of the present invention is achieved by adjusting the temperature and time of the annealing
15 and extension step among program setting values as set forth below.

Annealing step : $X^{\circ}\text{C}$, 30 seconds, wherein the annealing temperature, $X^{\circ}\text{C}$, wherein annealing temperature is set to " a " $^{\circ}\text{C}$ increment per " b " cycle.

Extension step : 72°C , Y seconds, wherein the extension time is set to " c " second increment per " d " cycle.

20 The program setting values of the PCR, X , Y , a , b , c , and d are appropriately adjusted in accordance with the T_m of primers to be used, and the size of DNA which needs to be amplified. In particular, the adjustment is as follows.

X : set to a value lower than the lowest T_m value of the primers by

2°C

“a” and “b” : a is basically set as value of $(T_{m_max} - T_{m_min}) /$ number of total cycle. b is basically 1, but in the case that the “a” value for 1 cycle is too small to set in PCR equipment, the “a” value is adjusted by changing the “b” value (i.e. the number of cycles). For example, 0.05°C per cycle is changed to 0.2°C per 4 cycles.

Y : this value is initially set as the time required in synthesizing DNA of the shortest size among amplified PCR products which need to be amplified and is determined as follows.

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$$Y(\text{sec}) = L_{\min} / \text{rate of DNA synthesis velocity of taq DNA polymerase (bp/sec)}$$

In the above equation, L_{\min} indicates the smallest size(bp) of the PCR products, and the rate of DNA synthesis of taq DNA polymerase is determined empirically and is generally 13 bp/sec.

15 c (sec): this value is set as the time required for synthesizing the largest size of DNA of among amplified PCR products sufficiently at latter part of whole PCR cycles and is determined as follows.

$$c(\text{sec}) = [(L_{\max} - L_{\min}) / (\text{rate of DNA synthesis velocity of taq DNA polymerase; bp/sec})] / (\text{total cycles} - 7)$$

20 In the above equation, L_{\max} indicates the largest size of the PCR products, and the L_{\min} indicates the shortest size of the PCR products.

“d”(cycle #) is basically 1. However, in the case that value of $L_{\max} - L_{\min}$ is not big so that the c value is too small to be set in the PCR equipment, this problem can be solved by changing the d(cycle) like the case

of changing the “a”.

Not only purified DNA (vector, genome DNA, cDNA library etc) but diluted blood can be used as samples for amplification by multiplex PCR method of present invention. If the blood is used as raw state, the PCR can not be properly performed due to proteins derived from plasma and erythrocyte. Therefore, the blood sample is prepared by dilution of 1X PCR buffer before PCR. About 1 : 30 (blood : buffer) is suitable as the dilution ratio, and diluted blood of 2 μ l is used for 50 μ l PCR. Concentrations of the other PCR elements are similar to general elements.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an electrophoresis picture of multiplex PCR product using diluted blood samples in a first embodiment of the present invention. (BF8, P5, BF5, in the order of size)

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Fig. 2 shows an electrophoresis picture of multiplex PCR products performed in a second embodiment of present invention using cloned BF5 and P5 of PCR products as templates, wherein the BF5 and P5 are cloned from PCR products primarily obtained from diluted bloods.

Fig. 3A shows the compared and analyzed result of a sequence of BF5 obtained from the first and second embodiments with its corresponding sequence of genbanks.

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Fig. 3B shows the compared and analyzed result of a sequence of P5 obtained from the first and second embodiments with its corresponding sequence of genbanks.

Fig. 3C shows the compared and analyzed result of a sequence of BF8 obtained from the first and second embodiments with its corresponding sequence of genbanks.

Fig. 3d shows the compared and analyzed result of a sequence of P1
5 obtained from the first and second embodiments with its corresponding sequence of genbanks.

Fig. 4A shows the picture of a fluorescent microscope for the P1 and P5 after performing a micro multiplex PCR in a silicon oxide chamber of a third embodiment.

10 Fig. 4B shows the picture taken of a fluorescent microscope after performing a micro multiplex PCR without P1 and P5 template DNAs in a silicon oxide chamber of the third embodiment negative control.

Fig. 5 shows a dimension of silicon oxide chamber used in the third embodiment.

15 Fig. 6A shows a CE (capillary electrophoresis) result of BR3 and BR4, amplified PCR products in a silicon oxide chamber of the fourth embodiment.

Fig. 6B shows a CE result of BR2 and BR3 amplified PCR products in a silicon oxide chamber of the fourth embodiment.

20 **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

Hereinafter, embodiments of the present invention will be explained with reference to the accompanying drawings. Although the present invention has been described in conjunction with the preferred embodiment, the present invention is not limited to the embodiments, and it will be apparent

to those skilled in the art that the present invention can be modified in variation within the scope of the invention.

First embodiment

Multiplex PCR of blood sample

5 Among exon sequences of brca1 and brca2 of human genomes which are related to breast cancer, and ovarian cancer, when mutation is occurred in the sequences, primers listed in table 1 were used to include sequences of products include sequences with high of cancer incidence. To include, in amplified products, sequences with high cancer incidence when mutated
10 among exon sequences of brca1 and brca2 of human genomes, which are related to breast cancer and ovarian cancer, respectively, primers listed in table 1 were used.

[Table 1]

prim er	Primer sequence	Tm (°C)	PCR product	
bf9	5'-TTTTAGCAAAAGCGTCCAGAA-3'	54.0	Brca 1 exon 11	BF 5
bf10	5'-TCGGTAGCAACGGTGCTATG-3'	59.4	Brca 1 exon 11	
p9	5'- TTTGATTACTCAGTTTAGAAAACC- 3'	55.3	brca 2 exon 11b	P5

p10	5'- GAAACATTCAGTTTTGTGCCCATGA GC-3'	61.6	brca 2 exon 11b	
bf15	5'- GTCCAATTTCAAATCACAGTTTTGG AGG-3'	62.2	brca 1 exon 11	BF 8
bf16	5'- TCTTTAAGATAGTCATCTGGTTTTCA GGCA-3'	62.7	brca 1 exon 11	
p1	5'- TGCTTGTGAATTTTCTGAGACGGAT G-3'	61.6	brca 1 exon 11	P1
p2	5'- AACAGAACTACCCTGATACTTTTCT GGA-3'	62.2	brca 1 exon 11	

Diluted blood was used as a sample for multiplex PCR. The sample was prepared as follows.

Total volume of PCR solution : 50 $\mu\ell$

Final concentration of each element

5 Primer concentration : 10pmol

MgCl₂ : 2mM

dNTP : 2mM

10 x PCR buffer : 5 $\mu\ell$

taq DNA polymerase : 2.5 units

diluted blood : 2. μl (dilution ratio – buffer:blood = 1:30)

pure water : up to 50 μl

The model “icycler” made from company Bio-rad has been used as a PCR equipment. This model comprises a controller capable of programming,
5 and the PCR parameters of the present embodiment are as follows.

1. preliminary denaturation step : 94 °C, 4 minutes

2. cycle step

denaturation step : 93 °C, 30 seconds

10 primer annealing step : 52 °C, 30 seconds (0.2 °C increment per cycle)

extension step : 72 °C, 15 seconds (1 second increment per 3 cycles)

40 cycles

3. additional extension step : 72 °C, 7 minutes

Fig. 1 shows picture of agarose gel after EtBr(Ethidium Bromide)
15 fluorescent treatment has been subject to, after the PCR solution amplified in the present embodiment had been subject to electrophoresis. Referring to Fig. 1, lane 1 indicates 100bp DNA ladder, and lane 3 indicates the result from multiplex PCR performed by the above procedure. BF5, P5, and BF8 correspond from the lowest arrow to the highest arrow, respectively. Lane 2
20 indicates the result from multiplex PCR performed with fixed conditions of primer annealing step and extension step such as a general PCR method. PCR conditions corresponding to the lane 2 are as follows.

1. preliminary denaturation step : 94 °C, 4 minutes

2. cycle step

denaturation step : 93 °C, 30 seconds

primer annealing step : 55 °C, 30 seconds

extension step : 72 °C, 40 seconds

40 cycles

5 3. additional extension step : 72 °C, 7 minutes

Identification of base sequences of the amplified products.

BF5(222bp), P5(317bp), and BF8(468bp) from the product (i.e. lane 3 of fig. 1) obtained in the first embodiment were separated from the agarose gel, and inserted to the Topo PCR2.1 cloning vector (made from the company, Invitrogen), and E. coli was transferred by introduction of the vector. The transformed E. coli was cultured and collected in LB(luria broth) containing dissolved, and collected, kanamycin (50 µg/ml), and each of transformed DNAs was purely refined from the E. coli, and base sequence for the DNAs was analyzed. PCR products were identified by comparing the analyzed base sequences to corresponding sequences of genbanks, and the results thereof were shown in Fig 3A to 3C.

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In the method of this embodiment wherein the diluted bloods were used as a template of multiplex PCR, the method can stay out of trouble of purely separating template DNAs, and the method of periodically changing conditions can also be more efficient than the conventional method performing PCR under fixed conditions. Even if the diluted bloods were used as a template, the remaining plasma protein and erythrocyte protein flowing during PCR treatment are obstacles to DNA amplification, so that when only leucocytes of bloods are partially refined or purely separated and subject to

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multiplex PCR, a better result is expected.

Second embodiment

Multiplex PCR of purely refined DNA

P1 and P5 were respectively subject to PCR using bloods diluted by
5 1:30 (blood : buffer) of 1 x PCR buffer. Cloning and base sequence analysis
were performed with the same method as that in the first embodiment (See Fig.
3-d, 3-b).

PCR elements of the same concentration and amount as those in the
first embodiment were put into one tube. Each DNA cloned with P1 and P5
10 was used with amount of 20ng as a PCR template instead of diluted blood.
Corresponding primer pairs were also put into the tube and finally the
sterilized water was prepared by the volume of the PCR mixture to reach 50
 μ l. Adjustment of parameters to PCR equipment and multiplex PCR
amplification reaction were performed in the same method as that in the first
15 embodiment. The specific adjustments are as follows.

1. preliminary denaturation step : 94 °C, 4 minutes

2. cycle step

denaturation step : 93 °C, 30 seconds

20 primer annealing step : 53 °C, 30 seconds (0.2 °C increment per
cycle)

extension step : 72 °C, 15 seconds (1 second increment per 3 cycles)

35 cycles

3. additional extension step : 72 °C, 7 minutes

Fig. 2 shows the electrophoresis picture of multiplex PCR products of

P1 and P5 amplified in this embodiment. In Fig. 2, lane 1 indicates the 50bp DNA marker, and lane 2 indicates the P1 and P5 which has been subject to multiplex PCR. The lower band among the two DNA bands is P1(127bp) and the upper one is P5(317bp).

5 Also, as performed in the first embodiment, P1 and P5 were purely separated and cloned, and then *E. coli* was transferred by introducing the vector. The transformed *E. coli* was then cultured and base sequence was analyzed. PCR products were identified by comparing the analyzed base sequences to its corresponding sequences within genbanks, and the results
10 thereof were shown in Fig 3B(i.e. result of base sequence analysis of P5) and 3d(i.e. result of base sequence analysis of P1).

In this embodiment, PCR parameters were set in accordance with the method of the present invention, and multiplex PCR was performed one time, thereby performing multiplex PCR for two DNAs could be successfully
15 achieved. However, by using refined DNA as a multiplex PCR template, a clearer result was obtained than by using diluted blood.

Third embodiment

Performing micro multiplex PCR in a silicon chamber

A revised quadranglar pyramid chamber of 0.2 μl volume was made
20 by KOH wet etching on a silicon wafer , and silicon oxides were deposited thereon by CVD (chemical vapor deposition) to make the surface thereof hydrophilic. 50 μl of PCR mixture like that in the second embodiment was made with, and 0.2 μl of the mixture was charged into the chamber and mineral oil was also put to cover the surface thereof so that the evaporation be

prevented. However, 1 x SYBR green I fluorescent destain material was contained in the PCR mixture. As the SYBR green I strongly emits fluorescence only when it is within the DNA of double chains, PCR amplification result can be verified. Therefore, when the PCR amplification
5 is not under proper performance, the fluorescence becomes unclear.

As a negative control, a sample was prepared from PCR mixture having all the same elements except a template DNA(i.e. vector DNA of which the P1 or P5 has been cloned).

Two silicon oxide chambers prepared as described above were
10 mounted on the upper side of the PCR tube hole of the PCR equipment (icycler from Bio-rad company), and then PCR was performed.

PCR conditions are set as below. As the thermal conductivity of the silicon material is higher than that of the PCR tube material, time adjustment of each step was set shorter.

- 15 1. preliminary denaturation step : 94℃, 4 minutes
2. cycle step
- denaturation step : 93℃, 15 seconds
- primer annealing step : 53℃, 15 seconds (0.2℃ increment per cycle)
- 20 extension step : 72℃, 15 seconds (1 second increment per 3 cycles)
- 35 cycles
3. additional extension step : 72℃, 3 minutes

The result of micro PCR was confirmed by the fluorescent microscope, and is shown in Fig. 4A and 4B.

Fig. 4A shows the result of multiplex PCR of P1 and P5 performed in a 0.2 μl silicon chamber, and Fig. 4B shows the result of negative control (i.e. multiplex PCR performed without a template). The fluorescence is barely seen in Fig. 4A, while it is strongly seen in Fig. 4A. Therefore, the multiplex PCR according to the method of the present invention can be successfully performed even with a sample having a small amount of 0.2 μl . Also, successful and normal PCR performance with small amounts of sample according to the present invention enables it useful to reduce the size of DNA amplification part within a gene diagnosis equipment.

10 Fourth Embodiment

Performing micro multiplex PCR in a silicon chamber(2)

In a silicon chamber of 1 μl , using genomic DNA as a template, a portion of breast cancer genome(brca1) including SNP was amplified by multiplex PCR, as in third Embodiment. The characteristics of primers used in these amplifications and PCR products were summarized in table 2.

【Table 2】

Primer	Primer Sequence	Tm(°C)	PCR Product(length)
br3	5' -6-fam-tccaagtacagtgagcacaattagcc	58	BR2(112base)
br4	5' -tggagcccacttcattagtactgg	57	
br5	5' -6-fam-ttgttctgagacacctgatgacctg	55	BR3(156base)
br6	5' -tgtgtatgggtgaaagggctagg	58	
br7	5' -6-fam-tccaagtacagtgagcacaattagcc	59	BR4(178base)
br8	5' -tggccctctgtttcttacctagttctg	62	

The primers were synthesized by adding a fluorescent material, 6-fam, at the 5'-terminal of forward primer.

The sequence of each amplified PCR product was determined by sequence analysis. The amplified PCR products were analysed by CE (capillary electrophoresis, ABI3700) and the results were depicted in Fig. 6a and 6b. Fig 6a is corresponding to BR3 and BR4, and Fig 6b is corresponding to BR2 and BR3.

The amplification condition for Fig 6a is set forth below.

Preliminary denaturation : 93 °C, 2minute and 30seconds

10 Denaturation : 93 °C, 20seconds

Primer Annealing : 53 °C, increased by 1 °C/5cycles

Extension : 72 °C, 12seconds, increased by 0.3seconds/5cycles
35cycles

Additional Extension : 72 °C, 7minutes

15 The amplification condition for Fig 6b is set forth below.

Preliminary denaturation : 93 °C, 2minute and 30seconds

Denaturation : 93 °C, 20seconds

Primer Annealing : 53 °C, increased by 1 °C/12cycles

Extension : 72 °C, 8 seconds, increased by 0.9seconds/7cycles

20 35cycles

Additional Extension : 72 °C, 7minutes

In the results of every CE analysis, the length of amplified PCR products are slightly different from the expected length. The differences were

caused by instrumental errors.

As described above, by changing the primer annealing temperature and extension time periodically, production of dimers from primers, and of non-specific product which has been the problems of conventional multiplex PCR can be prevented, and the PCR product can be obtained in shorter time by the method of present invention comparing to the conventional method wherein the optimal considerations of multiplex PCR were found by changing con. The multiplex products could be obtained in easier way by properly adjusting the increment or decrement of annealing temperature and extension time periodically.

Furthermore, the blood sample can be amplified through multiplex PCR in accordance with the present invention without refinement. Also, the multiplex PCR method of the present invention can be performed the PCR even with small amounts of sample less than 1 μl , so that the method enables it useful to use in the DNA diagnosis chip like lab-on-a-chip by which various PCR products should be obtained and analyzed in a small space.